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Effects of elevated pCO_2 on the nitrification activity of microorganisms in marine sediment

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Abstract

Nitrification is a process of nitrogen cycle, that is reacted by microbial organisms and is appeared anywhere in the sediment and water column in ocean environment. We assessed the effect of elevated pCO_2 on the nitrification of the sediment in Osaka bay, Japan. Ammonium oxidation activity among the nitrification process was suppressed by the elevated pCO_2. On the other hand the quantity and the diversity of *amoA* gene, which is the specific coding of the ammonium oxidation, were not affected by elevated pCO_2. Microbial activity such as ammonium oxidation could be a useful tool for detecting CO_2 leakage because of its common existence in the ocean.

Keyword Nitrification, *Ammonium oxidation*, *Sediment, amoA*, $^{15}N/^{14}N$ *ratio*

1. Introduction

Nitrogen cycle is one of the most important elemental flows in the ocean ecosystem because nitrogen is an essential element for various organic materials such as amino acid, DNA and so on. Nitrification, that is an oxidation process of ammonium to nitrate via amine monoxide and nitrite in the nitrogen cycle, is mainly active on the oxidative conditions such as the surface of sea bottom or water column. Especially the nitrification on the sea bottom is important to restore nitrogen settled in the sediment as the organic matter into the water column. It has been well known that nitrification was inhibited by lower pH in sewage or terrestrial soil. The suppression of nitrification with an acidification was recently reported in the Pacific Ocean or the inhibition of nitrification by the increasing of CO_2 concentration were reported in

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the ocean[1]. As nitrification is a common activity on the surface of sea bottom, its decline may become an useful index to detect CO_2 seapage. In this study, we assessed the effect of elevated CO_2 to the nitrification activity of the sediment in eutrophic bay and the biodiversity of *amo* gene, which is coded ammonium mono-oxygenase.

2. Materials and Method

2.1 Sampling

Samples used in experiments were collected from 20 m in depth of Osaka Bay, which is eutrophic and has a muddy sediment. Sediment cores (10 cm) were collected by diving for experiment 1 on autumn in 2010, and surface sediments were collected by smith-Mcintyre bottom sampler for experiment 2 on autumn in 2011(Fig.1).

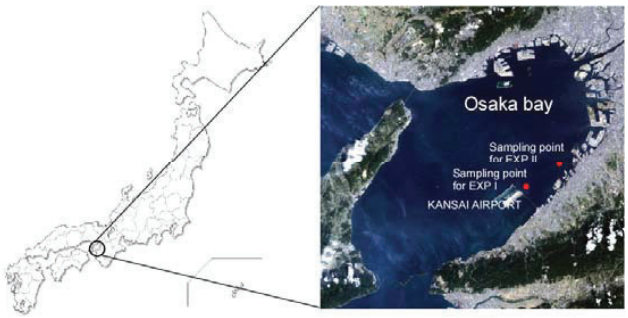

Fig.1 Sampling sites in Osaka Bay

2.2 Incubation Experiment

2.2-1 Experiment 1

Sediment cores were incubated at 23°C in dark for 28 days, sparging air/CO_2 mixing gases which adjusted CO_2 concentration at 1%, 5%, 20% and 50% into the upper water at 50 ml/min. Air was used in control. Nutrients concentration of upper water were analyzed by autoanalyzer (AACS-III BRAN-LUEBBE) at day 0, 3, 7, 14, and 28 of the incubation period. Each core was sectioned to 3 layers (0-0.5, 0.5-1, 1-2 cm). The ammonium oxidation rate and the biomass of microorganism were analyzed. The slurry of 0 - 0.5 cm layer of sediment of each core was poured in an incubation bottle for BOD testing, and then the bottle was filled by upper water of each core. The dissolved oxygen concentration measured by fluorescent DO sensor. Ammonium oxidation rate was estimated from the difference of the oxygen consumptions between the intact and the added condition with allylthiourea, which is the inhibitor of ammonium oxidation.

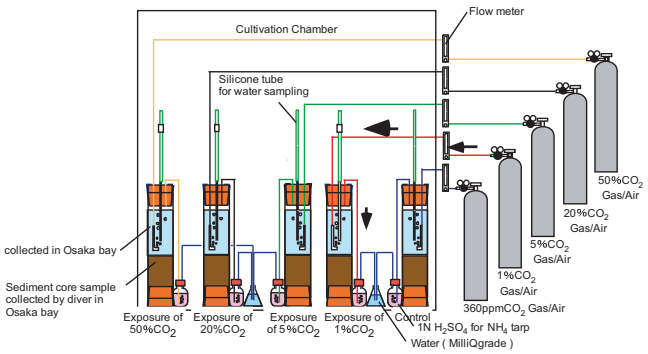

Fig.2 Incubation experiment layout for experiment 1

2.2-2 Experiment 2

Collected sediment was suspended with seawater and acclimated for 2 months in aerobic condition by sparging air to minimize the effect of heterotrophic oxidation. Some parts of acclimated slurry was diluted by seawater and incubated in sparging with air/CO_2 mixing gases (5%, 20% CO_2) and air as control for 48 hours at 23 °C in dark. And also, other slurries were adjusted at pH 6.5 and 5.9 respectively by adding 0.1 N HCl in sparging with air and incubated for 48 hours at 23 °C in dark. Sixty one ml of 10%(w/v) slurry adjusted with filtered seawater (0.22 µm) was putted into 500 ml glass vial and 615µL 20mM $^{15}NH_4Cl$ was added. The vial was sealed tightly. After 0,24,28 hours incubation, ^{15}N-labeled nitrate and nitrite was measured with GC-MS. Ammonium oxidation rate was calculated from the increase of $^{15}N/^{14}N$ ratio of nitrate and nitrite.

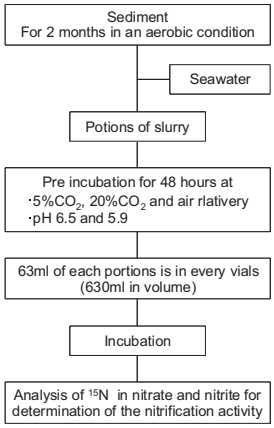

Fig.3 Assay scheme for experiment 2

2.3 Measurement of $^{15}N/^{14}N$ ratio

Suspended water of the incubated vial was filtered through a 0.2μm-pore-size polycarbonate filter. Nitrate in the filtrate was reduced to nitrite by cadmium-copper column. And then nitrite was reduced into N_2O by azide reduction method [4,5] and N_2O in headspace gas was measured by GC-MS QP-2010 (SHIMADZU).

2.4 DNA extraction.

For the qPCR and DGGE analysis, DNA was extracted from sediment by using ISOIL for Beads Beating (NIPPON GENE) according to the manufacture's direction. In Experiment 1, half grams of the sediment collected from 0-0.5 cm layer for each incubated core was applied. In Experiment 2, half grams of sediment from preincubation slurry was applied.

2.5 qPCR and DGGE analysis

Quantities of *Amo* gene of bacteria and archaea were analyzed respectively using qPCR method, and also the diversity of *Amo* was analyzed with the DGGE method. Archaeal *amoA* as well as bacterial *amoA*, gene copy numbers in all samples were determined in duplicate by using CFX96 Real time system (Bio-Rad Co., Ltd). PCR reaction run 35 cycles by using iQ supermix (Bio-Rad Co.,Ltd). Bacterial *amoA* gene were targeted for PCR using *amoA*-F1 (5'-GGGGTTTCTACTGGTGGT-3') /*amoA*-new (5'-CCCCTCBGSAAAVCCTTCTTC-3'). Archeae *amoA* gene were targeted using *amoA*-for (5'-CTGAYTGGGCYTGGACATC-3') /*amoA*-rev (5'-TTCTTCTTTGTTGCCCAGTA-3')[6,7]. Annealing temperatures and the primer combinations as listed in Table 1 and 2. Conversion of *amoA* to the number of cells for bacteria was calibrated from the qPCR analysis with *Nitrosomonas europea* known copy number of *amoA* gene. Number of cells for archaea was estimated on the assumption that one cells has a copy of *amoA* gene although archaeal *amoA* exist on one or more plasmids in a cell. Bacterial and archaeal *amoA* amplicons were analyzed by DGGE with 4 hr electrophoresis with the denaturant gradient of 20–50% for bacteria and of 30-50% for acrhaea respectively.

Table 1 PCR cycle for qPCR assay

	Bacteria	Archaea
1st Step（×1）	98℃、1min	98℃、1min
2nd Step（×35）	92℃、10sec 61.5℃、30sec(Plate Read) 72℃、1min	92℃、10sec 58.5℃、30sec(Plate Read) 72℃、1min
3rd Step（×1）	60℃、1min	60℃、1min
4th Step（×1）	Melt Curve 60℃ to 95℃:Increment 1.0℃ for 5sec (Plate Read)	Melt Curve 60℃ to 95℃:Increment 1.0℃ for 5sec (Plate Read)

Table 2 Constitution of PCR solution for qPCR assay

iQ Supermix	10μL
Primer set (10μM each)	1μL
templete ofDNA	1μL
distilled water	8μL
Total	20μL

3. Result

3.1 Experiment 1

Ammonium concentrations of upper water decreased in Control and 1% CO_2 condition, but increased in higher CO_2 concentrations more than 5%. Nitrite concentrations in higher CO_2 concentrations decreased in early stage during incubation and remain unchanged in latter.
Ammonium oxidation rate in less than 5% CO_2 was not inhibited at day 14 but those in more than 20% CO_2 were suppressed completely. Nitrification rate of the surface sediments incubated for 28 days was inhibited in more than 20% CO_2 as well as the day 14. However, nitrification in 1% and 5% CO_2 conditions declined as the increasing of CO_2 concentration compared with the control.

3.2 Experiment 2

Sediment exposed to 5% and 20% CO_2 for 48 hours lost ammonium oxidation activity, on the other hand sediments exposed to less than 5% CO_2 were not affected the activity. However, although the pCO_2 condition was consisted with air, sediments adjusted at pH 6.5 or 5.9 did not either kept normal activities. Number of *amo* gene can corresponds to the biomass of organisms operating ammonium oxidation. Although the activity of ammonium oxidation was inhibited at higher concentration of CO_2, the number of *amo* gene did not decrease during the incubation. Also, the biodiversities of *amo* gene were not changed significantly between control and higher CO_2 condition during the incubation.

4. Discussion

Nitrification is not a single reaction but a chain one composed of three enzymes. Ammonium oxidation is a key reaction affected by higher CO_2 condition among a series of enzyme functions (Fig. 4). This reaction is worked by an enzyme, ammonium mono oxygenase (*Amo*), and *amo* gene could shows good indexes of the biomass or species composition of nitrifying bacteria or archaea.
As nitrifying microorganisms grow slowly, a rapid change of the diversity is not expected for short incubation, therefore an adequate period (28 days) of incubation was applied on experiment 1. If high CO_2 concentration affected the diversity of nitrifiers, similar changes on *amo* gene diversity are expected. However, the band patterns of *amo* gene on DGGE analysis are not different among groups(Fig. 7). On the other hand, the ammonium oxidation activity disappeared early stage (Exp. 2). This result shows that the inhibition of ammonium oxidation caused by high concentration of CO_2 acts directly on the metabolic function but through the destruction of community or population structure microorganisms responsible to ammonium oxidation.
Ammonium mono-oxygenase use ammonium ion (NH_4^+) as a substrate, but there is little ammonium ion in lower pH. As a result, ammonium oxidation activity may be suppress. Since the ammonium mono-oxygenase and hydroxylamine oxidoreductase are located in membrane or periplasm, pH conditions in sea water may influence directly its activity. It is different from fish [8] or copepods [9] which are affected by partial pressure of CO_2.

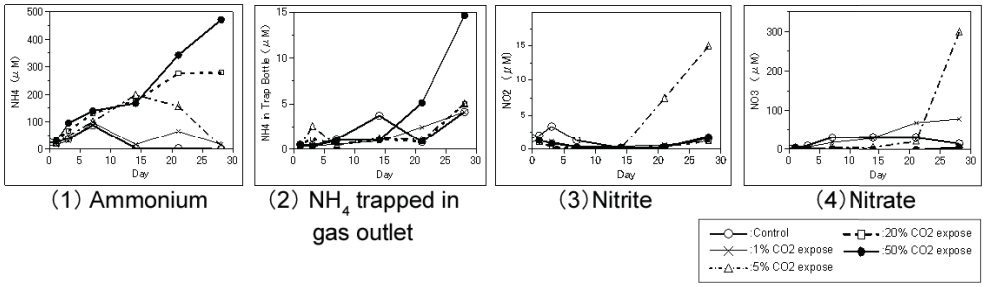

Fig.4 Change of nutrients concentration

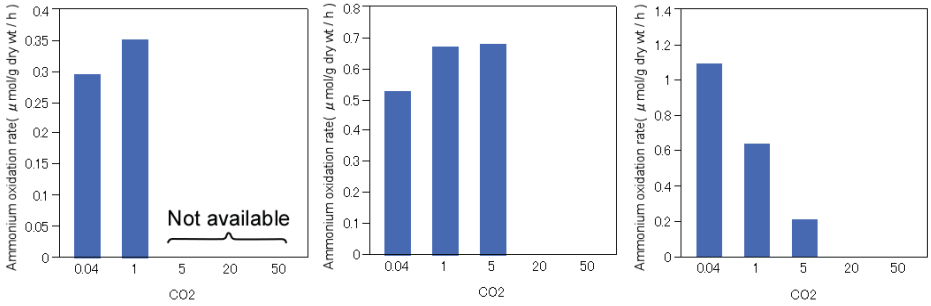

Fig.5 Ammonium oxidation rate (Exp 1)

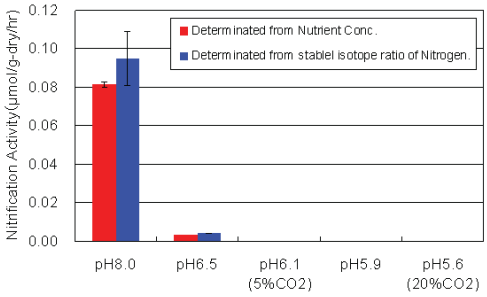

Fig.6 Nitrification activity rate (Exp 2)

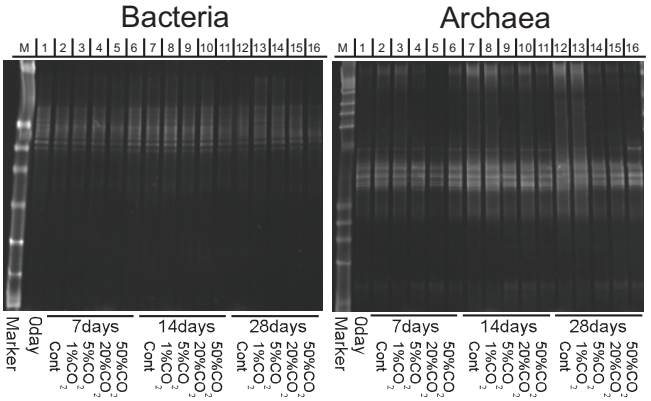

Fig.7 DGGE analysis of *amoA* transcripts after incubation

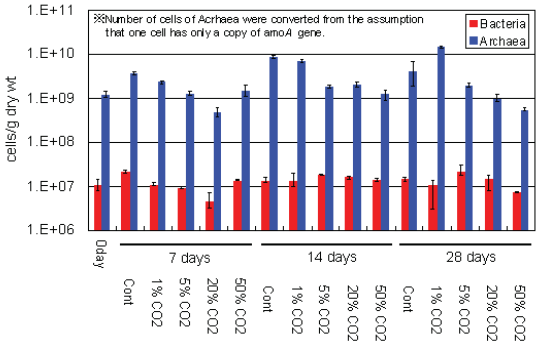

Fig.8 Copy numbers of archaeal and bacterial *amoA* were determined by qPCR

5. Conclusion

Ammonium oxidation activity was inhibited by higher concentration of CO_2 and lowering of pH. But either quantity and the diversity of *amoA* were not affected by CO_2 increasing. So we guess that higher concentration of CO_2 does not affect either growth or viability of nitrifier (bacteria and archaea), but affects only the ammonium oxidation metabolism. Therefore, when CO_2 concentration is back to a normal condition, ammonium oxidation activity recover certainly soon as before. We are studying the effect of enhanced CO_2 to the nitrification activities on oligotrophic Loch Lihne as a part of QICS experiment (Quantifying and Monitoring Potential Ecosystem Impacts of Geological Carbon Storage) in UK.

6. Acknowledgments

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